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Journal of Chromatography B, 812 (2004) 119–133
JOURNAL OF CHROMATOGRAPHY B
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Review

Analysis methods of ginsenosides

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Received 19 April 2004; accepted 20 July 2004
Available online 11 September 2004

Abstract

Ginsenosides are considered the main active principles of the famous Chinese traditional medicine "ginseng". For more than 30 years many researchers developed methods for the identification and quantification of ginsenosides in ginseng plant material, extracts and products. Separation of ginsenosides has been achieved using thin layer chromatography (TLC), gas chromatography (GC) and high performance liquid chromatography (HPLC). Among these techniques HPLC is by far the most employed. Ultraviolet (UV), evaporative light scattering (ELSD), fluorescence and, recently, mass spectrometry (MS) were coupled with HPLC for the detection of ginsenosides. The most recent methods are here discussed together with a critical evaluation of the published results. Furthermore new techniques such as near infrared spectroscopy (NIRS) and enzyme immunosassay (EIA) recently used for the determination of ginsenosides will be discussed.

Keyword: Ginsenosides

Contents

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doi:10.1016/j.jchromb.2004.07.039

1. Introduction

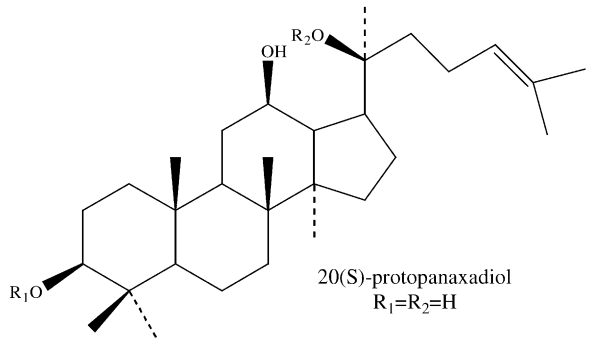
Ginsenosides are triterpenes saponins considered to be the main bioactive principles of the most important Oriental herbal medicine "ginseng" derived from the roots and rhizomes of different *Panax* species (Araliaceae). The most commonly used *Panax* species are *P. ginseng* (Korean or Asian ginseng), *P. quinquefolius* (American ginseng), *P. notoginseng* (Tienchi or Sanchi), *P. vietnamensis* (Vietnamese ginseng) and *P. japonicus* (Japanese ginseng). To date most of the research has focused on Korean ginseng which has been used in Asia for more than 5000 years as a tonic and a panacea that can promote longevity [1]. Nowadays Ginseng is used mainly to increase resistance to physical, chemical and biological stress and boost general vitality [2,3]. This activity of ginseng has been described as "adaptogenic" in most of the alternative medicine literature. Immume system modulation, antistress activities and antihyperglycemic activities are among the most notable features of ginseng in laboratory and clinical trials. Furthermore, a number of investigations points to antitumor properties and other pharmacological activities related to cancer, but no trials have confirmed a clinically significant anticancer activity yet [4]. Up to now more than 80 ginsenosides have been isolated from *Panax* species and most of them exhibit four types of aglycone moieties: protopanaxadiol, protopanaxatriol, ocotillol-type and oleanolic acid (Fig. 1). Four malonyl derivatives of ginsenosides Rb_1, Rb_2, Rc and Rd have also been described [5]. The malonyl derivatives and ginsenoside Ro are also called "acidic" ginsenosides while the other are usually named "neutral" ginsenosides. Due to the fact that Ginseng is a very popular phytomedicine used all around the world, a huge quantity of work has been carried out during the last 30 years in order to develop analytical methods for the identification, quantification and quality control of ginsenosides in raw plant materials, extracts and marketed products. One of the main goals of these researches was the differentiation of the ginsenosides pattern between the different *Panax* species in order to avoid adulteration or misidentification. Moreover, studies of changes in ginsenosides composition due to different traditional processing of *P. ginseng* roots such as white and red ginseng have been undertaken. Among all the classical techniques usually employed for phytochemical analyses high-performance liquid chromatography (HPLC) has been the method of choice for the analysis of ginsenosides in the last 20 years. The aim of the present paper is to highlight the most recent advances on the analysis of ginsenosides including hyphenated techniques and other techniques such as immunoassays and NIR.

2. Thin layer chromatography (TLC)

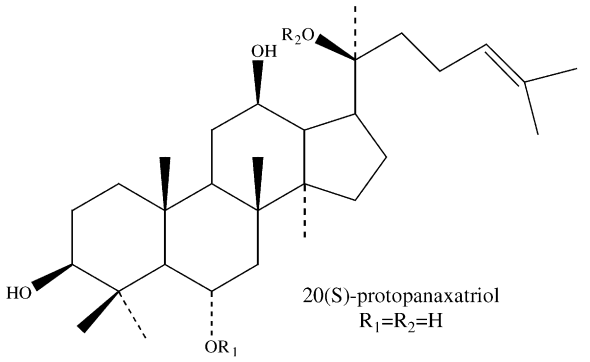
TLC is a very common technique for the fingerprint analysis of plant material and extract due to its easiness of use, low cost and versatility. Indeed, nowadays, United States Pharmacopoeia and European Pharmacopoeia still employ TLC for the identification tests of plant derivatives. Asian and American ginseng can be discriminated for their ginsenosides composition by two-dimensional TLC [6] using a mixture of chloroform, methanol and water (13:7:2) as the first developing solvent system (SS-I) and a mixture of water, *n*-butyl alcohol and ethyl acetate (5:4:1) as the second solvent developing system (SS-II). The plate is sprayed with freshly prepared anisaldehyde reagent. Ginsenosides F_{11}, Rg_1, Rg_2, Rf, Rc, Rd, Rc Rb_2, Rb_1 and Ro are separated (Fig. 2), in particular the presence of ginsenoside Rf is characteristic of Asian ginseng. With the introduction of densitometry, TLC has become also a useful tool for the quantitative analysis: Ginsenosides of *P. ginseng* roots and preparations can be quantified using HPTLC Silica gel F_{254} with a chloroform, ethyl acetate, methanol and water (15:40:22:9) developing system and revealed with anisaldehyde reagent [7]. Quantification of ginsenosides Ra, Rb_1, Rb_2, Rc, Re, Rd, Rg_1, Rf and Rg_2 is performed by remission absorption at 535 nm. Linearity, precision and accuracy of the method have been evaluated together with the detection limit (LOD for both Rb1 and Rg1 is about 10 ng/spot). Usually TLC detection of ginsenosides is achieved using anisaldehyde reagent which afford unstable colorations which limit the reproducibility and accuracy of the determination. Vanhaelen-Fastre et al. [8] employed vapours of thionyl chloride which allow the quantification of ginsenosides both in absorbance mode (275 nm) and in fluorescence mode (366 nm). The method developed has been shown to possess good selectivity, precision, accuracy and sensitivity for the determination of the six major ginsenosides Rb_1, Rb_2, Rc, Re, Rd, Rg_1.

3. Gas chromatography (GC)

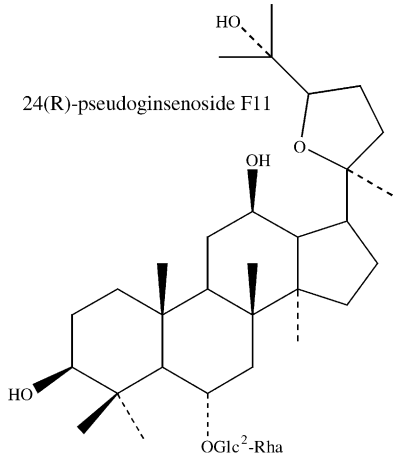
In the early eighties gas chromatographic separation of trimethylsilil (TMS) derivatives of ginsenosides in *P. ginseng* was developed. The saponins were identified by MS analysis. However, at that time the technology of the column was still in an early stage of development, indeed columns were in-house packed, for this reason the efficiency of the separation was not enough to separate Rg_1 and Rf and only seven ginsenosides could be detected and quantified [9,10]. More recently Cui et al. developed a new strategy for the analysis of ginsenosides in *P. ginseng* by GC-MS. Indeed they quantified the TMS derivatives of the aglycones 20(*S*)-protopanaxadiol 20(*S*)-protopanaxatriol and oleanolic acid after oxidative alkaline cleavage of the glycosidic chains (Fig. 3). Although this methodology does not allow to evaluate the complex pattern of ginsenosides, many interesting information have been obtained in these investigations [11–14]. Seventeen commercial ginseng preparations sold in Sweden were analysed for their content in ginsenosides. Furthermore, red ginseng and three liquid ginseng preparations were shown to contain significant amounts of 20-epimers of 20(*S*)-protopanaxadiol and 20(*S*)-protopanaxatriol as well



Ginsenoside	R_1	R_2
Rb_1	Glc^2-Glc	Glc^6-Glc
Rb_2	Glc^2-Glc	Glc^6-Ara(p)
Rc	Glc^2-Glc	Glc^6-Ara(f)
Rd	Glc^2-Glc	Glc
Malonyl-Rb_1	Glc^2-Glc^6-mal	Glc^6-Glc
Malonyl-Rb_2	Glc^2-Glc^6-mal	Glc^6-Ara(p)
Malonyl-Rc	Glc^2-Glc^6-mal	Glc^6-Ara(f)
Malonyl-Rd	Glc^2-Glc^6-mal	Glc
Rg_3	Glc^2-Glc	H
Rh_2	Glc	H
Rb_3	Glc^2-Glc	Glc^6-Xyl
Ra_1	Glc^2-Glc	Glc^6-Ara(p)^4-Xyl
Ra_2	Glc^2-Glc	Glc^6-Ara(f)^2-Xyl
Ra_3	Glc^2-Glc	Glc^6-Glc^3-Xyl
Rs_3	Glc^2-Glc^6-Ac	H



Ginsenoside	R_1	R_2
Rh_1	Glc	H
Rg_1	Glc	Glc
Rf	Glc^2-Glc	H
Rg_2	Glc^2-Rha	Glc
F_1	H	Glc
Notoginsenoside R_1	Glc^2-Xyl	Glc



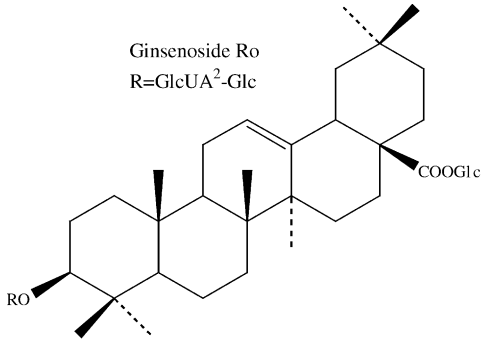
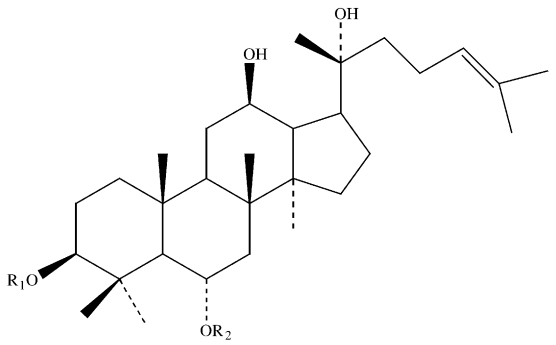
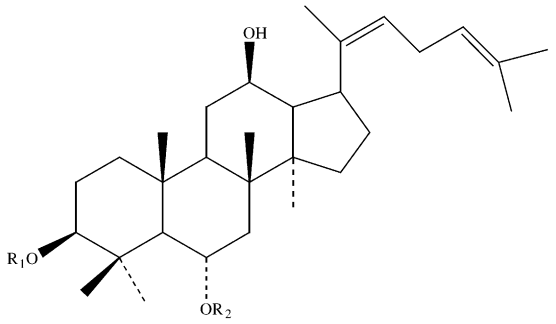


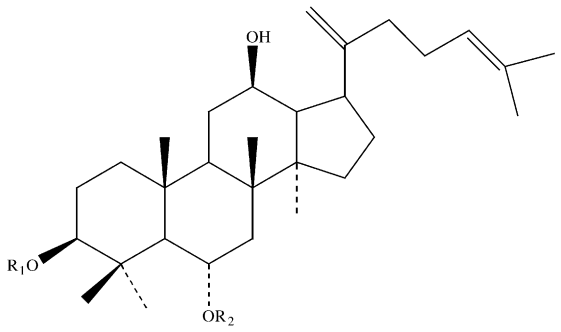
Fig. 1. Structures of the four main types of aglycone moieties and selected structures of ginsenosides. Glc, β-D-glucose; Rha, α-L-rhamnose; Ara(p), α-L-arabinose(pyranose); Ara(f), α-L-arabinose(furanose); Xyl, β-D-xylose; GlcUA, β-D-glucuronic acid; mal, malonyl; Ac, acetyl.



Ginsenoside	R_1	R_2
20(*R*)Rg_2	H	Glc^2-Rha
20(*R*)Rg_3	Glc^2-Glc	H
20(*R*)Rh_1	H	Glc
20(*R*)Rs_3	Glc^2-Glc^6-Ac	H



Ginsenoside	R_1	R_2
F_4	H	Glc^2-Rha
Rg_5	Glc^2-Glc	H
Rh_4	H	Glc
Rs_4	H	Glc^2-Glc^6-Ac



Ginsenoside	R_1	R_2
Rg_6	H	Glc^2-Rha
Rk_1	Glc^2-Glc	H
Rk_3	H	Glc
Rs_5	Glc^2-Glc^6-Ac	H

Fig. 1. (*Continued*).

as their corresponding 24,25-hydrated compounds. In addition two epimeric pairs of prosapogenins (ginsenoside Rg_3, 20(*R*)-Rg_3, Rh_1 and 20(*R*)-Rh_1) were also found in the liquid formulations suggesting that hydrolysis, epimerisation and hydration may occur in the liquid formulations under weak acidic conditions. The method was shown to be enough sensitive to allow the detection and quantification of ginsenoside aglycones in human urine samples after oral administration of ginseng preparations. Among 65 samples collected from athletes stating the consumption of *P. ginseng* preparations, 20(*S*)-protopanaxatriol could be found in 60 samples with a concentration between 2 and 35 ng ml^{-1} urine. The concentration of 20(*S*)-protopanaxadiol was always lower than 7 ng ml^{-1} urine. These findings would suggest that the uptake, metabolism and excretion of 20(*S*)-protopanaxadiol ginsenosides differ from that of 20(*S*)-protopanaxatriol ginsenosides in man.

4. High performance liquid chromatography (HPLC)

HPLC, because of its speed, sensitivity and adaptability to non-volatile, polar compounds, is ideal for the analysis of saponins and sapogenins. Another advantage is versatility due to the possibility of using different detection techniques such us ultraviolet (UV), evaporative light scattering (ELSD), fluorescence and mass spectrometry (MS).

4.1. Ultraviolet (UV)

Among the different techniques of detection of ginsenosides UV is the most employed since it is by far the most common detector found in phytochemical laboratories. Because of the weak UV absorption of ginsenosides, their detection is usually achieved at 198–205 nm. The great majority of the literature methods use C_{18} columns with water or phosphate

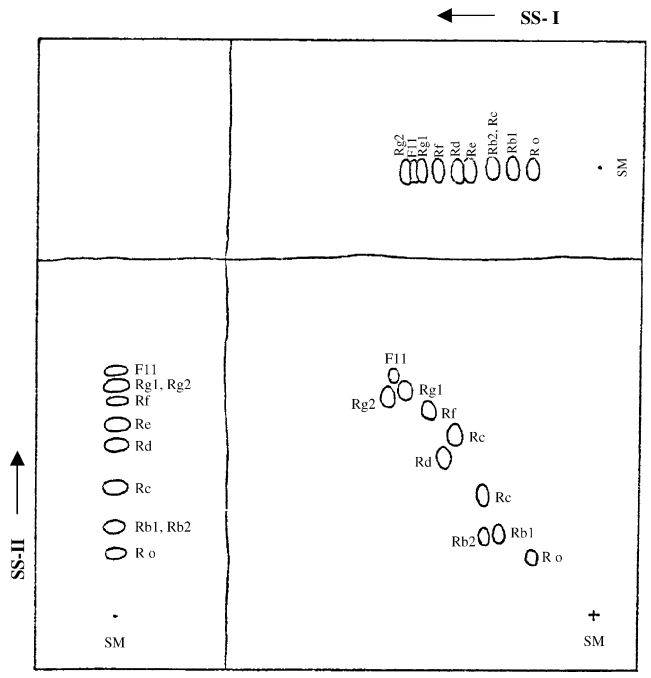


Fig. 2. One and two–dimensional TLC separation of ginsenosides Ro, Rb_1, Rb_2, Rc, Rd, Re, Rf, Rg_1, Rg_2, F_{11} standard mixture (SM). SS-I, first developing solvent system chloroform:methanol:water (13:7:2); SS-II, second developing solvent system water:*n*-butyl alcohol:ethyl acetate (5:4:1). Reproduced with permission of American Chemical Society and American Society of Pharmacognosy from [6].

buffers and acetonitrile mixtures as solvent system either in isocratic or in gradient elution mode [15–22]. The concentration of the phosphate buffer has been shown to be important in order to obtain separation of twelve ginsenosides in white *P. ginseng* extracts [23]. In particular troublesome separations

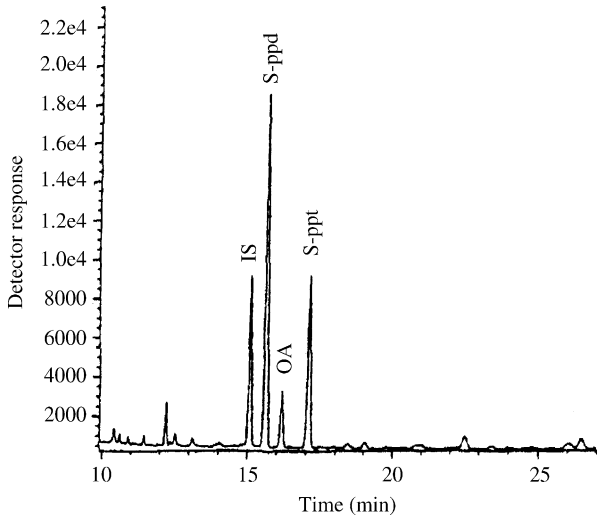


Fig. 3. GC profile of ginseng preparation after alkaline cleavage. Peak identity: IS, panaxatriol as internal standard; S-ppd, 20-(*S*)-protopanaxadiol; OA, oleanolic acid; S-ppt, 20-(*S*)-protopanaxatriol. Reproduced with permission of Elsevier Science from [12].

such as those of Rg_1 and Re, malonyl-Rb_1 and Rb_2, malonyl-Rb_2 and Rc were obtained using 10 mM KH_2PO_4 buffer (pH 5.88). Quantitation was performed using acetophenone as internal standard and the method was validated evaluating linearity, precision, accuracy and detection limit (0.2 μg for each ginsenoside). The same method was employed for a comparative study on 37 commercial samples of Ginseng radix [24] (Fig. 4). Content of ginsenosides Rb_1, Rb_2, Rc, Re, Rd, Rg_1, Rf, Rg_2 Ro, and three malonyl derivatives m-Rb_1, m-Rb_2 and m-Rc was determined for white and red *P. ginseng*, *P. quinquefolius* and *P. notoginseng* roots. This investigation showed that *P. notoginseng* possesses the highest content in ginsenosides followed by *P. quinquefolius*, roothair of *P. ginseng* and finally red and white ginseng. Red ginseng was shown to be different from white ginseng due to the lack of malonyl ginsenosoides. Differently from white ginseng, which is obtained from the dried roots of *P. ginseng*,

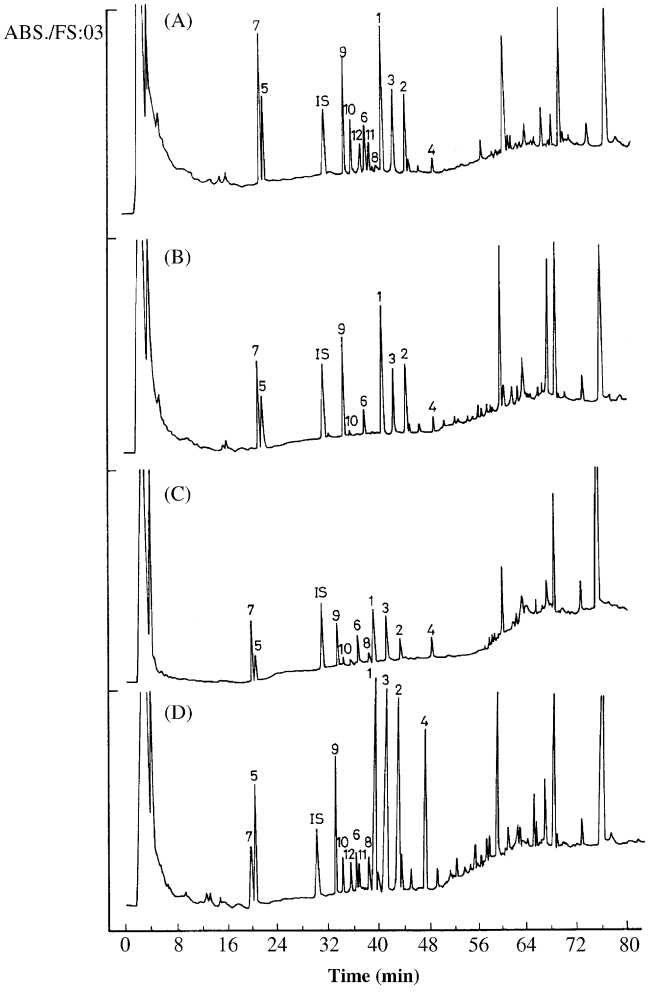


Fig. 4. HPLC chromatograms of ginseng extracts. (A) white ginseng, (B) red ginseng, (C) shihchu ginseng, (D) Asian ginseng hairy roots, (E) wild American ginseng, (F) cultivated American ginseng, (G) Sanchi ginseng. Peaks identity: (1) Rb_1, (2) Rb_2, (3) Rc, (4) Rd, (5) Re, (6) Rf, (7) Rg_1, (8) Rg_2, (9) Ro, (10) mRb_1, (11) mRb_2, (12) mRc. Reproduced with permission of Thieme from [24].

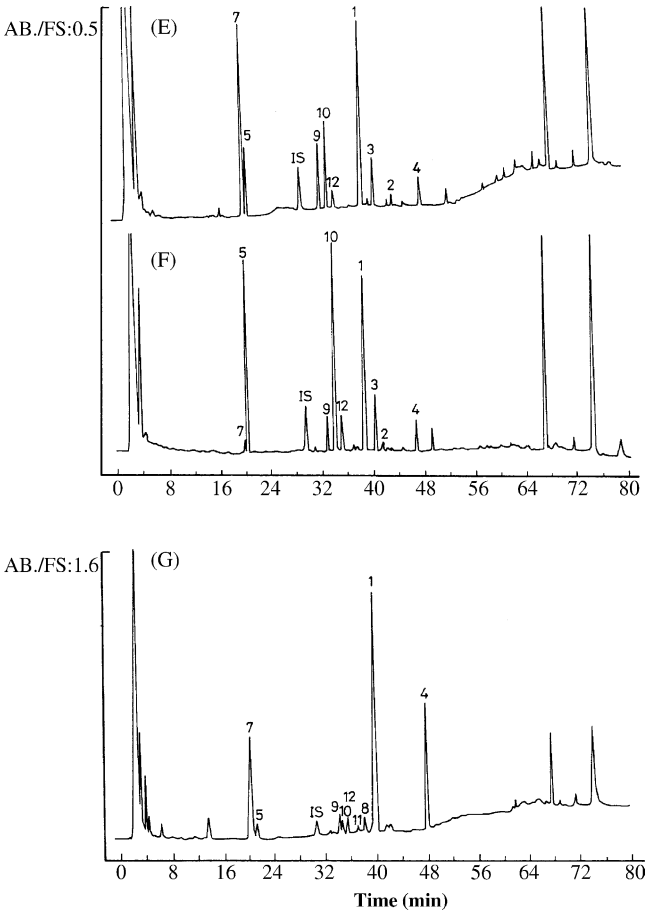


Fig. 4. (*Continued*).

red ginseng is produced by steaming and drying up the roots of *P. ginseng*. This heating procedure causes degradation of the malonyl ginsenosides m-Rb_1, m-Rb_2 and m-Rc and m-Rd in the ginsenosides Rb_1, Rb_2, Rc and Rd, respectively. American ginseng does not contain Rf and possesses a ratio Rg_1/Rb_1 much lower than *P. ginseng*. Traditionally the ratio Rg_1/Rb_1 is linked to the ethnopharmacology properties of ginseng preparations [1]. Indeed, since Rb_1 was shown to act as weak CNS depressant while Rg1 stimulates the CNS, the low ratio of Rg_1/Rb_1 is linked to the so-called "cool" or calming properties of American ginseng, while the high ratio Rg_1/Rb_1 could be the reason of the "warm" or stimulating characteristics of Asian ginseng [4]. Finally, ginsenoside Rb_2 was not found in *P. notoginseng* roots. Further studies using the HPLC method previously described were undertaken in order to evaluate the effect of the drying conditions on the content of ginsenosides in red ginseng [25]. Compared with the traditional hot air drying, a new combined microwave-hot air method showed a substantial decrease (approximately 30–40%) in drying time and had little influence on the ginsenoside contents and the colour of the final product. Samukawa et al. [26] developed a HPLC-UV method for the determination of the minor degradation products of ginsenosides formed during the steaming process of red ginseng 20(*R*)-Rg_2, 20(*R*)-$Rh_1$20(*S*) and 20(*R*)-Rg_3, together with other eighteen ginsenosides originally present in white ginseng. The separation was performed on a Superspher RP-18 (e) column using a gradient of 0.1%H_3PO_4–H_2O–acetonitrile (8:72:21, v/v) and acetonitrile in 120 min together with a step gradient of temperature (0–30 min: 35 °C, 30–60 min:55 °C, 60–120 min:35 °C). *P. notoginseng* is also found on the market in the raw and steamed forms. The raw material has been widely used in Chinese medicine for its hemostatic and cardiovascular properties while the steamed form was claimed to nourish blood and to increase production of various blood cells in anaemic conditions. A method which allows the differentiation of raw and steamed form was developed using a Waters Symmetry C_{18} column with a gradient of water and acetonitrile [27]. The content of notoginsenoside R_1, ginsenosides Rg_1, Re, Rb_1 Rc and Rd was evaluated showing a decrease upon steaming. Furthermore, the chromatogram of the steamed material showed several new peaks in the region between 60 and 80 min and the authors postulated them to be the degradation products occurring during the steaming procedure. One of the main problems in the analyses of ginseng is the determination of malonyl-ginsenosides due to the lack of suitable standards because of the high instability of these compounds. Court and Hendel [28] developed an indirect method for the quantification of acidic saponins in American ginseng roots exploiting the characteristic of malonyl-ginsenosides to degrade into the corresponding neutral ginsenosides upon hydrolysis with aqueous potassium hydroxide. The analyses were performed on a Waters Resolve C_{18} column using a ternary gradient of phosphate buffer (pH 5.81)–acetonitrile–water and *m*-cresol as internal standard.

4.2. Evaporative light scattering detector (ELSD)

The main problems encountered in performing HPLC-UV analyses of ginseng are the high level of baseline noise and the poor sensitivity due to the weak UV absorption of ginsenosides. This feature also limits the choice of solvents and mobile-phase modifiers for improved separation. ELSD is a mass detector which measures the scattered light generated by the non-volatile particles of analyte produced by the nebulization into droplets of the LC effluent. The signal intensity is related to the concentration of the solute in the effluent but not its chemical identity. ELSD is a universal, non-specific detector which can provide a stable baseline even with gradient elution. Furthermore, a number of volatile mobile-phase modifiers, such as NH_4OH, $(C_2H_5)_3N$, NH_4OAc, HCOOH, CH_3COOH, CF_3COOH, can be used in order to obtain better selectivity. Important parameters to be settled for optimised detector response are the flow rate of the nebulizer gas (nitrogen) and the drift tube temperature. However, these parameters are influenced by the configuration of the instrument which varies depending on the producer, hence the published conditions are often to be adjusted prior the analysis. Another feature of ELSD is that linear calibration curves are

generated by plotting log-transformed peak area under curve versus log-transformed concentration of the standards. White and red ginseng have been analysed by HPLC–ELSD for their content in ginsenosides Rb_1, Rb_2, Rc, Re, Rd, Rg_1, Rf, Rg_2, Rg_3 and Rh_1 using a LiChrosorb NH_2 column and gradient elution of mixtures of acetonitrile–water–isopropanol A (80:5:15) and B (80:20:15). The minimum detectable concentration was reported to be more than 35 ng of ginsenosides on column [29]. The same method was used to evaluate whether steaming Asian ginseng at high temperatures can enhance the yield of red ginseng specific ginsenosides Rg_5, Rg_3 and F_4 [30]. These products have great importance for the biological activity of ginseng since Rg_3 showed strong vasorelaxation properties and anti-platelet aggregation activity while Rg_5 exhibited anti-cancer activity through the induction of apoptosis. Ginsenosides F_4, Rg_3 and Rg_5 which were absent in raw ginseng, were detected after steaming. In particular Rg_3 and Rg_5 were the most abundant in the material steamed at 120 °C, accounting for 39 and 19% of total content of ginsenosides, respectively. The same research group developed a new HPLC–ELSD method for the determination of less polar ginsenosides in processed *P. ginseng* [31]. The separation and the detection of F_4, Rg_3, Rg_5, Rg_6, Rk_1, Rk_3, Rs_3, Rs_4, Rs_5 together with the 20-(*R*) epimers of Rg_2, Rh_1, Rg_3 and Rs_3 was achieved using a C_{18} column with a acetonitrile–water–acetic acid gradient (Fig. 5). This method does not separate Rg_1 and Re, however, the quantification of seven genuine ginsenosides and 15 degradation product specific of steamed *P. ginseng* was achieved. Four acidic and 13 neutral saponins in Asian ginseng roots and extracts were successfully separated on a reversed-phase C_{18} column with 8 mM ammonium acetate (pH 7 with ammonium hydroxide) and acetonitrile as the mobile phase in a linear gradient program [32]. Detection was performed using both UV at 203 nm and ELSD. However, ELSD was chosen for the quantitative determination because it showed a better sensitivity and no drift of the baseline (Fig. 6). The method developed was proven to provide exhaustive extraction for ginseng roots and accurate and precise data for both acidic and neutral saponins. Identification of ginsenosides and evaluation of the specificity of the method was performed by means of HPLC–ESI-MS and MS^2 analyses [33]. Recently, a minor ginsenoside, 24(*R*)-pseudoginsenoside F_{11}, described in American ginseng, has been reported to improve memory performance. Li and Fitzloff [34] developed a fast HPLC–ELSD method for the determination of 24(*R*)-pseudoginsenoside F_{11} in American ginseng by means of Waters Spherisorb ODS-2 C_{18} column using acetonitrile and water under gradient conditions. Comparison between UV and ELSD detection showed very poor UV absorption even when the injection amount was increased to 1060 ng on column while the detection limit of ELSD was observed at 53 ng. Another comparative study between UV detection and ELSD was performed for the HPLC analysis of ginsenosides Rb_1, Rb_2, Rc, Re, Rd and Rg_1 in American and Asian ginseng [35]. Separation was achieved on Waters Spherisorb ODS-2 C_{18} column using acetonitrile and water under gradient conditions over 80 min. The two detection techniques were found to be comparable with regard to sensitivity, calibration and reproducibility using these chromatographic conditions.

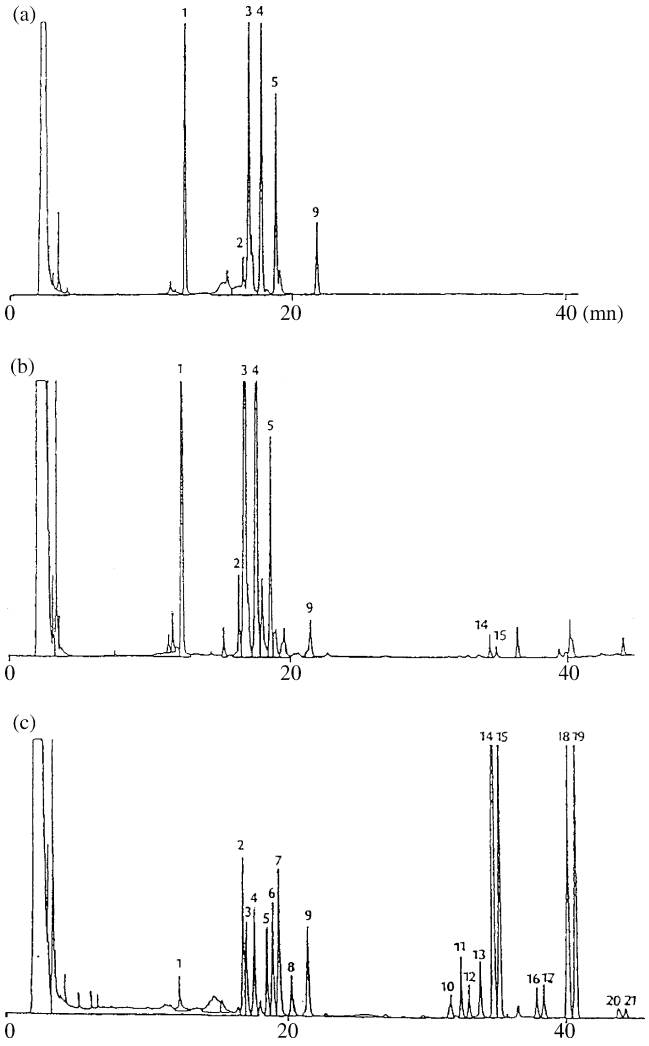


Fig. 5. HPLC scaled chromatograms of fresh *P. ginseng* (a), steamed *P. ginseng* at 100 °C/3 h (b), steamed *P. ginseng* at 120 °C/3 h (c). Peaks identity: (1) Re + Rg_1, (2) Rf, (3) Rb_1, (4) Rc, (5) Rb_2, (6) Rg_2, (7) 20-(*R*)-Rg_2 + Rh_1, (8) 20-(*R*)-Rh_1, (9) Rd, (10) Rg_6, (11) F_4, (12) Rk_3, (13) Rh_4, (14) Rg_3, (15) 20-(*R*)-Rg_3, (16) Rs_3, (17) 20-(*R*)-Rs_3, (18) Rk_1, (19) Rg_5, (20) Rs_5, (21) Rs_4. Reproduced with permission of Elsevier Science from [31].

4.3. Fluorescence

Fluorescence is one of the most sensitive detection methods for HPLC analyses. However, since ginsenosides do not possess a suitable fluorescence chromophore they have to be derivatized before detection. A HPLC method using photoreduction fluorescence detection was described for the analysis of ginsenosides Rb_1, Rb_2, Re, Rd and Rg_1 in *P. ginseng* and *P. notoginseng* [36,37]. Ginsenosides were separated on an amino column using acetonitrile and aqueous

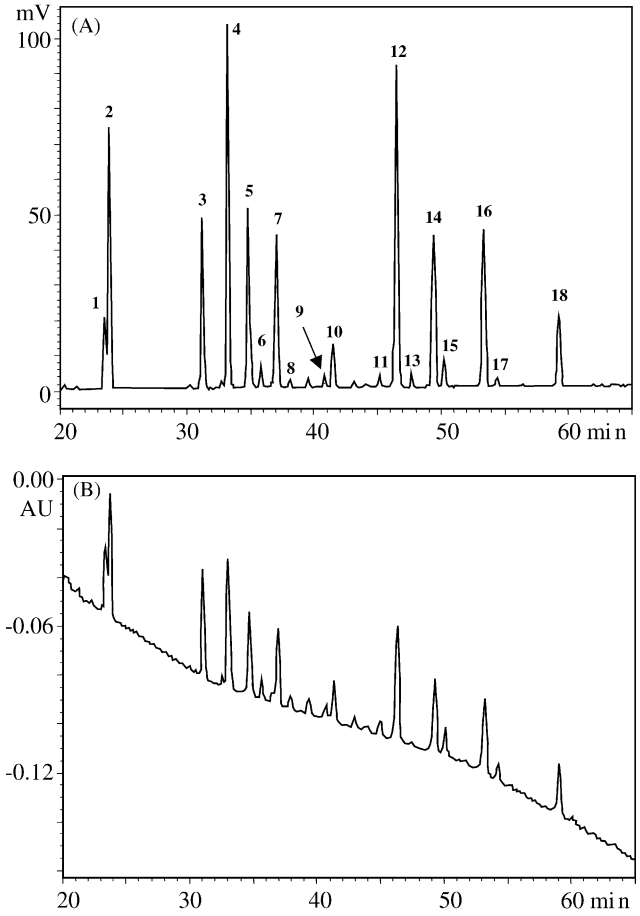


Fig. 6. Comparison between HPLC–ELSD (A) and HPLC-UV (B, detection λ = 203 nm) chromatograms of *P. ginseng* roots: 8.0 g of plant material were extracted with 100 ml of aqueous ethanol (40%, v/v). Peaks identity: (1) Rg_1, (2) Re, (3) Ro, (4) malonyl-Rb_1, (5) malonyl-Rb_2/Rb_3/Rc, (6) malonyl-Ra_1/Ra_2, (7) malonyl-Rb_2/Rb_3/Rc, (8) Ra_3, (9) Rf, (10) malonyl-Rd, (11) malonyl-Rd isomer, (12) Rb_1, (13) Rg_2, (14) Rc, (15) Ra_1/Ra_2/isomer, (16) Rb_2, (17) Rb_3, (18) Rd.

2-*tert*-butylanthraquinone (t-BAQ) solution. The column effluent was passed through a 45-cm PTFE capillary tube coiled around a 10 W-UV lamp to reduce t-BAQ to a highly fluorescent dihydroxy anthracene derivative of ginsenosides which was detected by a fluorescence detector (excitation: 400 nm, emission: 525 nm). This method showed good selectivity for ginsenosides and sensitivity comparable to that of UV detection (around 50 ng). Recently a novel pre-column derivatization method for the quantitative determination of ginsenosides Rb_1 and Rg_1 by HPLC with fluorescence detection was developed [38]. The double bond at the C_{24}–C_{25} position of ginsenoside was converted into aldehyde group by means of ozonolysis. Reaction of the aldehyde group with 9-fluorenylmethoxycarbonyl (FMOC) hydrazine formed the ginsenosides FMOC-hydrazone which were separated by HPLC on a Waters Nova-Pack C18 column using methanol–water–0.1%TFA linear gradient. The detection was performed by fluorescence (excitation at 270 nm, emission at 310 nm) allowing to achieve a detection limit for ginsenosides Rb_1 and Rg_1 of 2 and 1 ng, respectively.

4.4. Mass spectrometry (MS)

In the last few years HPLC–MS techniques have been successfully applied to the analysis of non-volatile molecules such as saponins. HPLC combined with a frit-fast atom bombardment (FRIT-FAB) [39,40] and thermospray (TSP) [41] interfaces were used for the qualitative analysis of *P. ginseng*. The major drawbacks of these interfaces were a poor reproducibility, which prevents their use for quantitative analyses, and a high fragmentation in the recorded spectra. Furthermore, these techniques did not allow the identification of the thermally unstable malonyl-ginsenoside. The electrospray ionization (ESI) interface was, recently, introduced as a highly sensitive and soft ionization technique for the HPLC–MS analysis of thermolabile molecules. This technique provides structural information about molecular weight and, using collision-induced dissociation (CID) experiments, aglycone type and the masses and sequences of the sugar residues. A number of works were performed on ginsenosides in order to study the influence of ionization mode, mobile-phase modifier and metal ions adducts on the sensitivity of the analysis and the structural information obtained [42–46]. In general, ion sensitivities for the ginsenosides were greater in the negative ion mode, but more structural information on ginsenosides was obtained in the positive ion mode. The negative ion mode ionization of ginsenosides afforded the $[M - H]^-$ ion together with several adduct ions depending on the mobile-phase modifier. The product ion mass spectra of $[M - H]^-$ showed signals due to the successive loss of sugars from the glycosidic chains with the most intense signals due to the monosaccharides ions (Fig. 7). In the positive ion mode $[M + H]^+$ ions were observed together with the cationized ions $[M + Li]^+$, $[M + Na]^+$ and $[M + K]^+$. These have been shown to be particularly useful for the determination of the ginsenosides structure. Indeed the CID mass spectra of the molecular and cationized ions exhibited also signals characteristic of the sugar chains such as ions retaining the charge on the sugar moieties after glycosidic cleavages from the triterpene core and cross-ring cleavages of the sugar residues (Fig. 8). HPLC–ESI-MS and MS/MS analysis of *P. ginseng* roots extract allowed the identification of 25 ginsenosides [33]. Among the identified constituents several minor ginsenosides were detected which were not described previously, in particular, two isomers of ginsenosides Ra_1 and Ra_2 and seven malonyl-ginsenosides. However, this technique does not allow the structural identification of the isomers. The chromatographic method was previously described in the ELSD section [32] and detection was operated in negative ion mode. The ESI-MS spectra of neutral ginsenosides, exhibited the quasi-molecular ion $[M - H]^-$, together with adduct ions $[M + AcO]^-$ and $[M - CH_2O + AcO]^-$ and double-charged adduct species such as $[M - H + AcO]^{2-}$ and

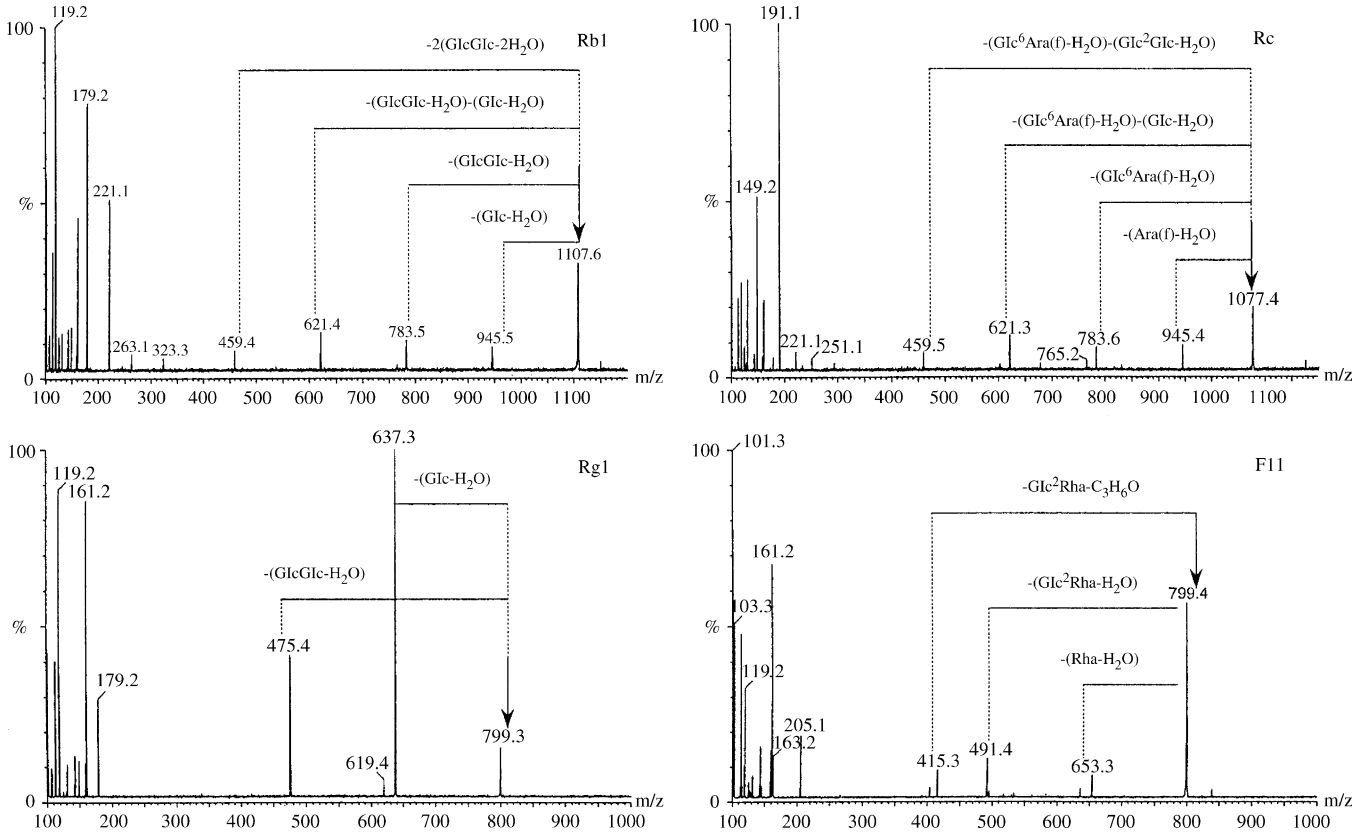


Fig. 7. Product ion mass spectra of $[M - H]^-$ from ginsenosides Rb_1, Rg_1, Rc, and F_{11}. Arrows show the mass-selected ion for MS/MS measurement. High intensity ions at *m/z* 149, 161 and 179 correspond to $[Ara - H]^-$, $[Glc - H_2O - H]^-$ and $[Glc - H]^-$ ions, respectively. Electrospray ionization mass spectrometry of ginsenosides, Miao et al. Copyright © 2002 John Wiley & Sons. Reproduced with permission from [45].

$[M + 2AcO]^{2-}$. Malonyl-ginsenosides exhibited the quasi-molecular ion $[M - H]^-$ together with quasi-molecular ion $[M - CO_2 - H]^-$, adduct ions $[M - CO_2 + AcO]^-$ and strong double-charged adduct species such as $[M - CO_2 - H + AcO]^{2-}$ and $[M - CO_2 + 2AcO]^{2-}$. The presence of the latter is due to the thermal instability of malonyl-ginsenosides. Indeed acidic ginsenosides decomposed in the interface capillary (t = 290 °C) losing CO_2 from the malonic acid unit. The MS/MS spectra exhibited a fragmentation pattern corresponding to the successive loss of the glycosidic units including the $[Aglycon - H]^-$ ions. HPLC–MS/MS in negative ionisation mode was also performed for the quantification of ginsenosides Rb_1, Rb_2, Rc, Re, Rf, Rd and Rg_1 in commercial samples of *P. ginseng* and *P. quinquefolius* [47]. Separation was performed on a narrow bore Zorbax C_{18} column with water and acetonitrile as mobile phase in gradient conditions. Under these conditions Rg_1 and Re co-eluted but they could be quantified separately using the difference in molecular ions and product ions. Indeed concentrations of the seven ginsenosides were determined by peak area of the most abundant product ions. HPLC–MS/MS in positive ion mode was employed for the determination of ginsenosides Rb_1, Rb_2, Rc, Re, Rf, Rd and Rg_1 in plant extract of Asian and American ginseng [48]. A fast separation (20 min) was obtained on a narrow bore YMC ODS-AQ column using water acetonitrile 0.01% acetic acid in gradient conditions. Quantification was performed by selected reaction monitoring (SRM) choosing $[M + H]^+$ as the precursor ions and monitoring the most abundant fragment ion which was a di-saccharide ion for ginsenosides Rb_1, Rb_2, Rc, Re and Rd and the $[Aglycone + H - 3H_2O]^+$ ion for Rf and Rg_1. The detection limit for ginsenosides was 2 pg on column. Confirming the results presented previously, American ginseng was shown to contain a higher amount of ginsenosides of protopanaxadiol group than Asian ginseng, while the opposite occurs for the protopanaxatriol group. HPLC–MS/MS was used to distinguish *P. ginseng* and *P. quinquefolius* through the detection and the quantification of ginsenoside Rf and 24(*R*) pseudoginsenoside F_{11} [49,50]. Li et al. found that 24(*R*) pseudoginsenoside F_{11} was present in abundance in American ginseng (more than 0.1%, w/w) while only 0.0001% (w/w) was detected in Asian ginseng. Ginsenoside Rf was only found (more than 0.021%, w/w) in Asian ginseng. Hence contamination of American ginseng by Asian ginseng would be evident by the presence of Rf (Fig. 9). Furthermore the ratio Rf/F_{11} (not less than 700) may be used to identify Asian ginseng and to determine whether it is contaminated with American ginseng. Recently, HPLC–MS and MS/MS analyses of malonyl-ginsenosides

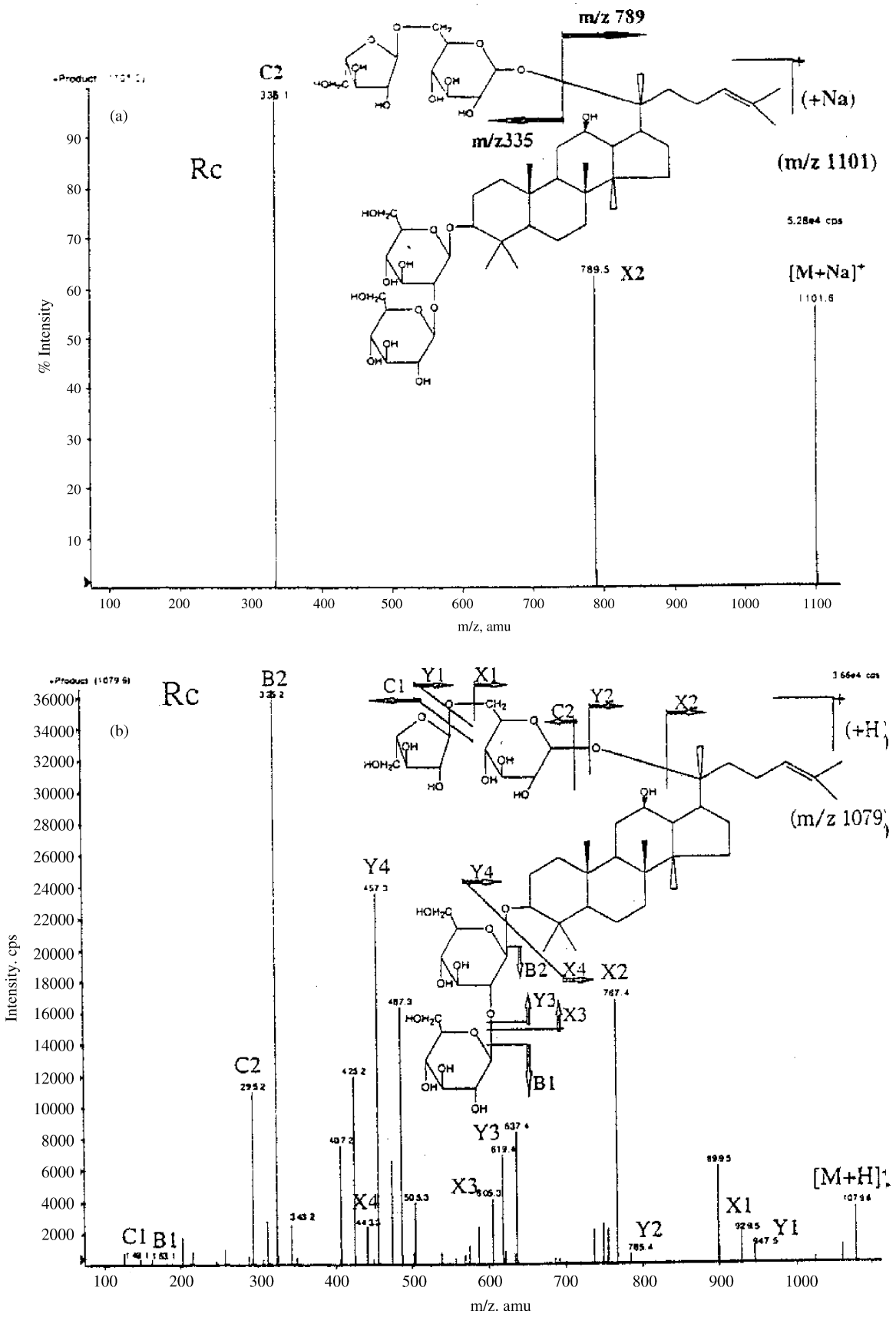


Fig. 8. Product ion mass spectra of $[M + Na]^+$ (a) and $[M + H]^+$ (b) ions from ginsenoside Rc. Reproduced with permission of American Chemical Society from [48].

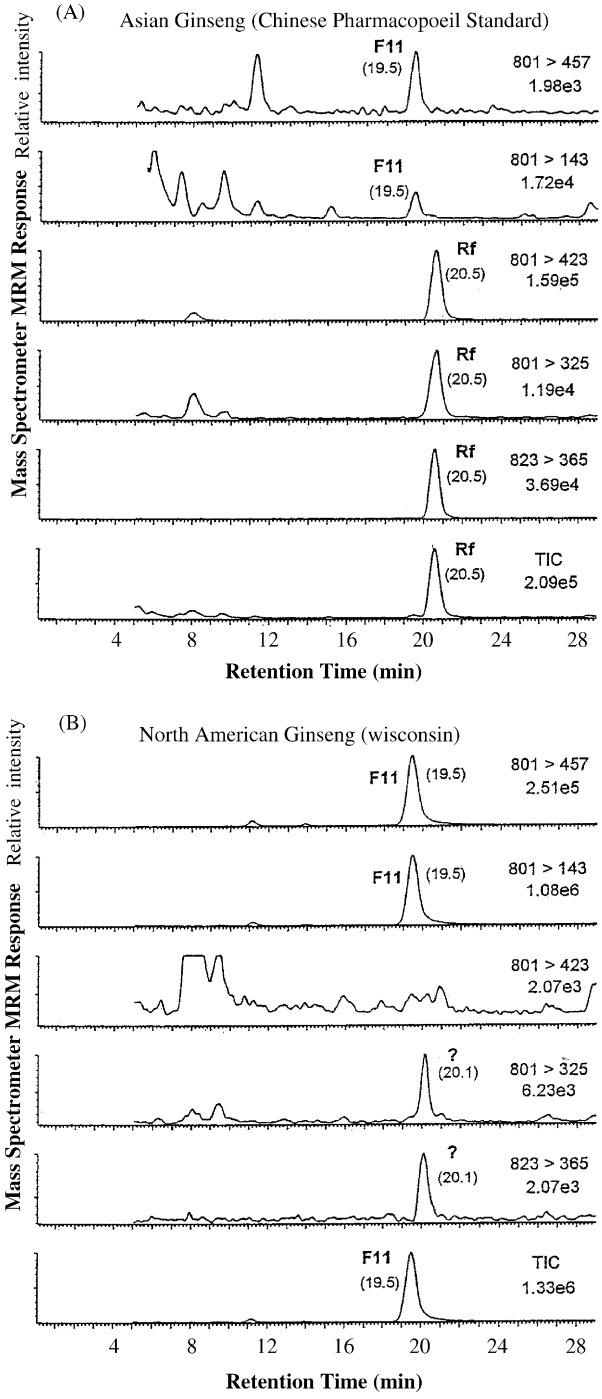


Fig. 9. HPLC–MS/MS Multiple Reaction Monitoring (MRM) chromatograms of Asian ginseng (A) and North American ginseng (B). Precursor/product ion pairs selected for MRM of Rf: $[M + Na]^+$ 823/$[GlcGlcNa]^+$ 365, $[M + H]^+$ 801/$[MH - GlcGlc - H_2O]^+$ 423, $[M + H]^+$ 801/$[GlcGlc - H_2O + H]^+$ 325. Precursor/product ion pairs selected for MRM of F_{11}:$[M + H]^+$ 801/$[MH - RhaGlc - H_2O]^+$ 457, $[M + H]^+$ 801/$[C_8H_{15}O_2]^+$ 143. ? = unknown ginsenoside. Reproduced with permission of American Chemical Society from [50].

have been proposed to differentiate *P. ginseng*, *P. quinquefolius* and *P. notoginseng* [51]. The method developed was based on the peculiar characteristic of malonyl-ginsenosides to loose CO_2 from the malonyl group upon CID. Indeed, acidic ginsenosides could be revealed in the chromatogram by displaying a total ion profile of those MS/MS scans that showed a neutral loss of 44 Da. Using this technique the authors could detect several acidic derivatives, in particular two malonyl-ginsenosides of the protopanaxatriol group, m-Rg_1 and m-Re, not previously described in *P. ginseng*. American ginseng was shown to possess a level of m-Rc and m-Rb_2 relative to m-Rb_1 lower than Asian ginseng. *P. notoginseng* did not show the presence of m-Rc and m-Rb_2, the three most abundant acidic ginsenosides being m-Rb_1 and two isomers of m-Rb_1 (Fig. 10).

5. Capillary electrophoresis (CE)

Due to the absence of charge in ginsenosides, capillary zone electrophoresis (CZE) was not applicable. Therefore, micellar electrokinetic chromatography (MEKC) was employed for analysis of ginsenosides Rb_1, Rb_2, Re, Rc, Rf, Rd and Rg_1 in *P. ginseng* extract [52]. The analysis was performed using 100 nm l^{-1} borate containing 80 mmol l^{-1} cholate, pH 10 and a capillary length of 75 cm using chloramphenicol as inernal standard. The voltage and capillary temperature was fixed at 30 kV and 30 °C, respectively. The standard ginsenosides were separated in 20 min and detected using UV at 200 nm (Fig. 11). The analysis of the extract showed that ginsenoside Rf co-elute with other components. Validation was performed for the quantification of ginsenoside Rb_1 evaluating linearity, precision and accuracy.

6. Sample extraction

A wide variety of procedures has been employed for the extraction of ginsenosides from plant material. Most methods used methanol or ethanol or different aqueous mixtures of these two solvents. However, the use of methanol–water mixtures, instead of pure methanol, has been shown to provide enhanced extraction performances for the isolation and purification of ginsenosides [53]. Extraction trials were carried out at room temperature or using heat [29–31,54] or sonication [28,32,35,55] to enhance recovery of ginsenosides. The use of heat in the extraction procedure was proved to degrade the thermally unstable malonyl-ginsenosides into the corresponding neutral ginsenosides. Court et al. [28] showed that, while partial degradation (50%) occurred after 5 h of extraction using methanol in soxhlet apparatus, a minimum of 20 h were necessary to achieve total conversion. A simple method using ethanol–water 40:60, sonication for 15 min followed by shaking for 4 h was developed and validated for the simultaneous quantification by HPLC–ELSD of acidic and neutral

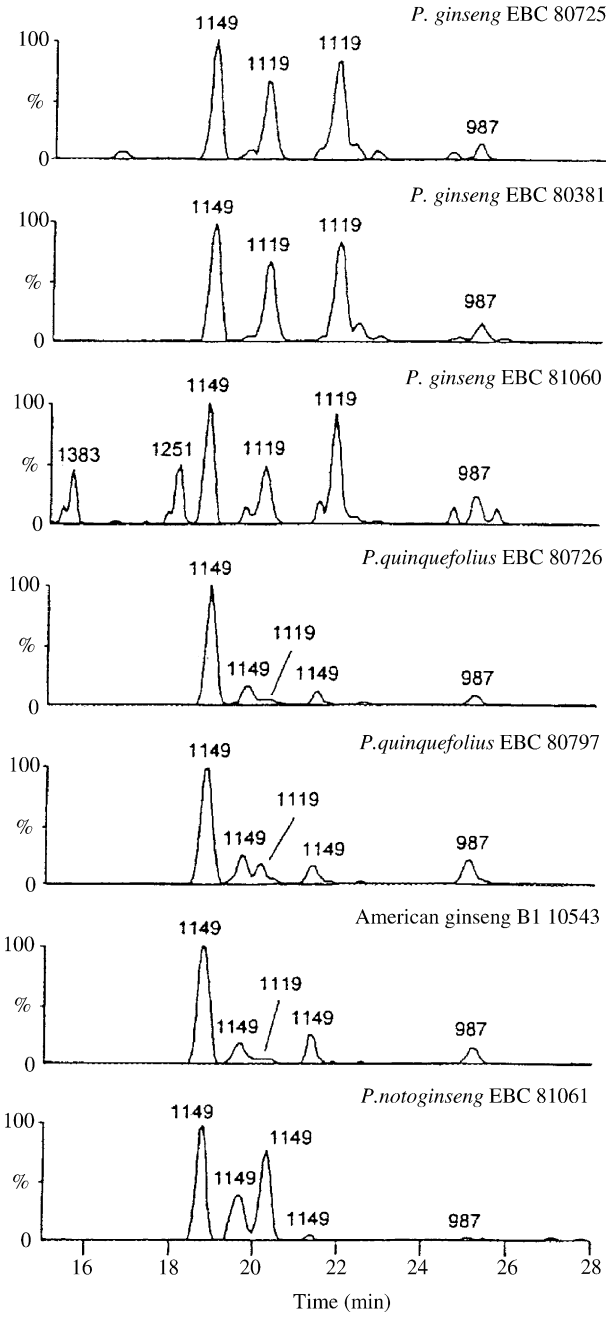


Fig. 10. HPLC–MS/MS chromatograms of protopanaxadiol malonyl-ginsenosides in Asian and American ginseng plant material and extracts. Numbers above peaks are the *m/z* values of the base ion in the MS/MS spectrum corresponding to $[M - H - CO_2]^-$. Liquid chromatography/mass spectrometry of malonyl-ginsenosides in autentication of ginseng, Kite et al. Copyright © 2002 John Wiley & Sons. Reproduced with permission from [51].

ginsenosides in *P. ginseng* roots [32]. Extraction using sonication (3 × 30 min) in methanol was employed to evaluate the quality of commercial drugs of Asian and American ginseng for their content in ginsenosides Rb_1, Rb_2, Rc, Re, Rg_1 and Rd [35,55]. Comparison with an extraction procedure which employed methanol–water 30:70 at 50 °C for 30 min

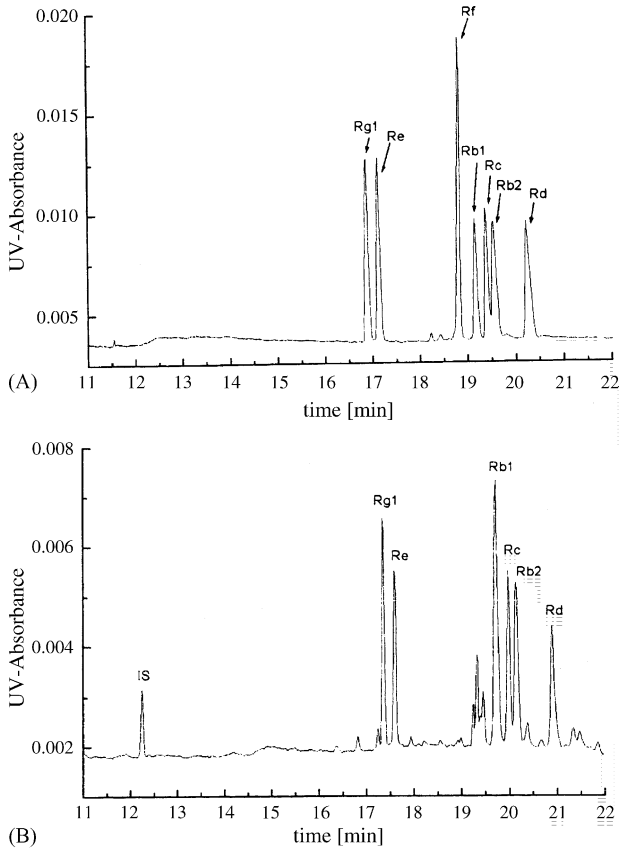


Fig. 11. (A) MEKC separation of seven standard ginsenosides. (B) MEKC chromatogram of *P. ginseng* roots extract using aqueous ethanol (50%, v/v). IS, chloramphenicol. Reproduced with permission of Thieme from [52].

showed that the latter was less efficient for the recovery of ginsenosides than the other [55]. The same authors developed and validate specific extractions methods for the analyses of softgels, fluid extracts, tablets and caplets.

7. Near infrared spectroscopy (NIRS)

NIRS has been successfully applied for food composition analysis, food quality assessment and in pharmaceutical production control. The main advantages of this technique over the traditional chemical and chromatographic methods are the rapidity and the easiness of use in routine operations. Moreover, NIRS is a non-destructive technique which does not require sample preparation. However, for quantitative analyses, the instrument has to be calibrated using a set of samples (20–50) with known analyte concentrations obtained by suitable reference methods. Typical NIRS procedures start from the collection of NIR reflectance spectra over the 400–2500 nm spectral region of a suitable number of samples with known value. Calibration model are produced using multiple linear regression (MLR), partial least squares (PLS) or principal component regression (PCR). Data pre-treatment using mathematical transformation of the NIR spectra can be

applied to enhance spectral features and/or remove or reduce unwanted sources of variation during the development of the calibration models. Simultaneous quantification of ginsenosides Rb_1, Rb_2, Rc, Re, Rd, Rg_1, Ro, m-Rb_1, m-Rb_2, m-Rd and m-Rc in American ginseng roots was performed by NIRS [56]. Twenty-six samples of American ginseng roots were analysed for their content in ginsenosides by HPLC-UV and, for each sample, NIR spectra were collected over 400–2500 nm. The HPLC and spectral data obtained were used for building the calibration equation and performing the cross validation. Several mathematical treatments (first, second and third derivatives of log 1/reflectance) and statistical models (PCR, PLS and MPLS) were evaluated in order to obtain the best calibration equation. Among the calibration equations for the 11 individual ginsenosides, those of ginsenosides Rb_1, Re and m-Rb_1 showed the lowest relative standard deviation. The same procedure was applied for the determination of Rb_1, Rb_2, Rf, Re, Rd and Rg_1 in *P. notoginseng* [57]. These investigations showed that the accuracy and precision of NIRS methods for the ginsenosides quantification are comparable with those obtained with HPLC. The main drawback of this technique is the calibration step, which requires analyses of several samples covering all the expected spectral variability of the sample and a reliable HPLC method for the determination of ginsenosides as a reference method for the calibration.

8. Enzyme immunoassay (EIA)

Enzyme immunoassay (EIA) using both polyclonal [58–60] and monoclonal antibodies (MAb) [61,62] was developed for the qualitative and quantitative determination of ginsenosides. The first step for the development of EIA method was the synthesis of a hapten–carrier protein conjugated. Bovine serum albumin (BSA) conjugated of ginsenosides Rb_1, Rg_1, Rf, F_1 and Rg_2 were produced and employed for the elicitation in rabbit of polyclonal antibodies or for the preparation of specific MAb in mouse. Recently a simple procedure using periodate oxidation was developed for coupling ginsenosides with BSA [59]. Both the synthetic hapten–carrier protein conjugates and the MAb produced were characterized by matrix-assisted laser desorption/ionization (MALDI) mass spectrometry [62]. Ginsenoside Rf was analysed using a competitive and indirect EIA [59]. The EIA involved competitive inhibition by Rf of the binding of the anti-Rf polyclonal antibody to an ovalbumin (OVA)-Rf solid phase coating antigen on a microtiter plate. The binding of the polyclonal antibody to the well was monitored using a peroxidase-labeled anti-rabbit IgG. With this method Rf could be determined over the range 10 pg–10 ng. Furthermore, the assay showed little cross reactivity with ginsenosides Rb_1, Rh_1, Rh_2, Rg_1, Rg_3, while exhibited high cross-reactivity with Rg_2. Recently a highly sensitive and specific Enzyme linked immunosorbent assay (ELISA) method was developed for the determination of 20(*S*)-protopanaxatriol ginsenosides (PPT) [60]. Polyclonal antibodies raised against ginsenoside F_1-BSA showed high reactivity to PPT, and minor reactivities to other ginsenosides. Using ELISA, the working range extends from 50 pg ml^{-1} to 20 ng ml^{-1} and the method was proven to be a useful tool for the determination of PPT in biological fluids. Two MAb against Rb_1 and Rg_1 were produced using hapten–carrier protein conjugates with BSA and employed for ginsenosides analyses using Western blotting methodology [63,64]. Western blotting using MAb is a common assay methodology for the detection of high molecular weight substances. Ginsenosides were separated on a TLC plate, blotted to a polyvinylidene difluoride (PVDF) membrane and fixed by forming a ginsenoside–BSA conjugate through consecutive treatment with $NaIO_4$ and BSA. Staining of ginsenosides was achieved by incubating the membrane with anti-ginsenoside Rb_1 and Rg_1 MAb followed by peroxidase-labeled goat anti-mouse IgG in the presence of various substrate. Blue color bands appeared for protopanaxadiol ginsenosides after treatment using 4-chloro-1-naphthol while purple spots were observed for protopanaxatriol ginsenosides with 3-amino-9-ethyl carbazole. The detectable limit for Rb_1 using Western blotting was 360 pmol. Although this technique was shown to be specific and sensitive for the determination of ginsenosides, it remains too elaborate for routine analysis of ginseng. Interestingly, this methodology has allowed the direct immunocytolocalization of ginsenosides directly in fresh *P. ginseng* roots, thus showing that the highest content of ginsenosides is found primarily in the endodermis cells, followed by the exodermis tissue and the radial vascular bundle [61].

9. Evaluation of the analytical results

Ginseng comprises one of the largest sales categories of commercial herbal products in the world. Ginseng products are sold in capsule, liquid extract, softgel, tablet and tea forms mainly produced using *P. ginseng* and *P. quinquefolius*. Ginsenosides are widely considered as the main active compounds of ginseng and are used as markers for the quality control of ginseng drugs and commercial products. Total content in ginsenosides and Rb_1/Rg_1 ratio are used for the standardization of ginseng products. In particular, ratios differed among species: Rb_1/Rg_1 values usually between 1 and 3 are characteristic of *P. ginseng*, while Rb_1/Rg_1 values around 10 or greater are indicative of *P. quinquefolius*. Also, the presence or absence of marker compounds is used for species differentiation. The absence of ginsenoside Rf is used to identify *P. quinquefolius* and to exclude adulteration. Reviewing the huge amount of literature produced about the analytical methods for ginsenosides, one can notice different quantitative results depending on the employed methodology. Total ginsenosides content in *P. ginseng* varied from 0.2 to 2% for main roots and from 4 to 9% for root hair. *P. quinquefolius* roots have been showed to possess a total

ginsenosides content ranging from 4 up to 10%. In order to evaluate the quality of the products on the US market, the American Botanical Council (ABC) has started the Ginseng Evaluation Program (GEP) in 1993 [65]. The part one of GEP presented data on the evaluation of consistency of standardized *P. ginseng* products through the HPLC-UV quantification of ginsenosides Rb_1, Rb_2, Rc, Re, Rg_1 and Rd. The GEP found substantial variation in the use of the term "standardized". For instance some products employed the term "standardized" with no claim about the content in ginsenosides of the extract. Other products were standardized to contain a certain percentage of ginsenosides, usually 4–7%. The results of GEP showed that the majority of standardized products analysed met the minimal standards of quality control. However, the GEP recommended that a more complete labelling should be introduced in order to clarify the ginsenosides dose that could be expected per unit. Furthermore, an acceptable range of ginsenosides content should be established by the industry, FDA and/or U.S. Pharmacopoeia. Recently, U.S. Pharmacopoeia published monographs for *P. quinquefolius* and *P. ginseng* roots and extract [66]. The total content in ginsenosides, calculated as the sum of ginsenosides Rb_1, Rb_2, Rc, Re, Rg_1 and Rd and determined by HPLC-UV, is not less than 4% for *P. quinquefolius* roots and 10% for the extracts. *P. ginseng* roots were defined to contain not less than 0.2% Rg_1 and 0.1% Rb_1 using the same method as *P. quinquefolius*. A different HPLC-UV method is employed for the analyses of *P. ginseng* extracts which contains not less than 3.0% of ginsenosides Rb_1, Rb_2, Rc, Re, Rg_1 and Rd. European Pharmacopoeia published a monograph on *P. ginseng* roots in which the content of ginsenosides Rb_1 and Rg_1, determined by HPLC-UV, is not less than 0.4% [67]. Concerning the literature data, besides the natural variation due to the heterogeneity of the plant material, the main reason of this variability of ginsenosides content is attributable to the choice of the compounds to be quantified in ginseng. Some authors quantified neutral ginsenosides Rb_1, Rb_2, Rc, Re, Rg_1 and Rd stating that they make up 90% of total saponin content [35,55]. However, other authors showed that the content of acidic saponins malonyl-ginsenoside Rb_1, Rb_2, Rc, and Rd represent between 35 and 60% of the total content of ginsenosides in both *P. ginseng* and *P. quinquefolius* [24,28]. Since malonyl-ginsenosides are likely to release ginsenosides upon consumption of ginseng products the content of sole neutral saponins may not reflect the potency of the product. Furthermore, the relative contents of acidic and neutral ginsenosides could be used for the determination of the age and the processing of ginseng samples.

10. Conclusions

HPLC-UV is the commonly used method for the quantification of ginsenosides in plant material, extracts and marketed products. The choice of this technique is mainly due to the large availability of HPLC-UV instrumentation in analytical laboratories. However, because of the weak UV absorption of ginsenosides, detection is performed at 200–205 nm producing chromatograms with high level of baseline noise and, consequently, poor sensitivity. ELSD was proven to be a valuable alternative detection method for the HPLC analysis of ginsenosides which produces stable base line chromatograms and allows to extend the choice of solvent systems for enhanced chromatographic separation. A limitation of both UV and ELSD detection is the lack of information on the identity of chromatographic peaks that is usually obtained by injection of standard compounds. The coupling of mass spectrometer with HPLC allowed the on-line identification of ginsenosides producing important structural information such as molecular weight, sugar unit sequence and aglycone moiety. Hence, ginseng drugs derived from different *Panax* species were differentiated on the basis of their ginsenosides distribution by application of HPLC–MS and MS/MS methodology. Furthermore, HPLC–MS and MS/MS techniques demonstrated to be a highly sensitive and specific analytical methods for the quantification of ginsenosides. However, even if this methodology is still too expensive to be used in routine analyses it remains an essential tools for farmacokinetics and metabolism studies. Major drawbacks of the use of HPLC for routine analyses of ginsenosides are time consuming sample preparations and long analyses times (usually more than 60 min), due to the presence of the high number of constituents to be separated. NIRS seems to be the technique of the future for the routine analyses of ginsenosides. NIRS was applied with success in the determination of ginsenosides in plant material showing a precision and accuracy comparable with HPLC. This technique is rapid, does not need extensive sample preparation and is simple to use in routine operations. However, the instrument has to be calibrated on several samples with known ginsenosides concentrations obtained with a suitable reference method. Concerning the quantitative data, a great variability in the content of ginsenosides is found in literature. This variability can be in part ascribed to natural variations such as kind of soil, weather conditions, geographical location and different production procedures. However, these sources of variability should be minimised with the introduction of good agricultural practices (GAPs) and good manufacturing practices (GMPs). Furthermore, the divergence in the reported levels of ginsenosides among investigators can also be attributed to different sample handling, such as extraction, different testing methodologies and interpretation. In particular many researchers do not take into account malonyl-ginsenosides which were demonstrated to represent up to 60% of the total content in ginsenosides. Since ginseng is one of the most sold oriental herbal medicine in the world, it is important that the national authorities adopt common analytical methodologies in order to establish precise quality standards and have a control on the marketed products. A first step has been taken by both USP and European Pharmacopoeia which have recently introduced monographs for *P. ginseng* (USP and Ph. Eur.) and *P. quinquefolius* (USP) plant material and extract. However, a

harmonisation of specifications and analytical methodologies has still to be reached and would be highly welcome.

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